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MINI REVIEW

Chondrodysplasias due to proteoglycan defects

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The proteoglycans, especially the large chondroitin sulfate proteoglycan aggrecan, have long been viewed as important components of the extracellular matrix of cartilage. The drastic change in expression during differentiation from mesenchyme to cartilage, the loss of tissue integrity associated with proteoglycan degradation in several disease processes and, most important, the demonstration of abnormalities in proteoglycan production concomitant with the aberrant growth patterns exhibited by the brachymorphic mouse, the cartilage matrix deficient mouse, and the nanomelic chick provide the strongest evidence that the proteoglycan aggrecan is essential during differentiation and for maintenance of the skeletal elements. More recently, mutations associated with proteoglycans other than aggrecan, especially the heparan sulfate proteoglycans, glypican and perlecan, suggest an important role for these molecules in skeletal development as well. This review focuses on the molecular bases of the hereditary proteoglycan defects in animal models, as well as of some human chondrodysplasias, that collectively are providing a better understanding of the role of proteoglycans in the development and maintenance of the skeletal elements.

Key words: aggrecan/chondrogenesis/chondrodysplasia/glypican/perlecan

Introduction

Proteoglycans are a family of complex macromolecules characterized by the presence of one or more glycosaminoglycan (GAG) chains covalently linked to a polypeptide backbone. Although originally named and categorized on the basis of the GAG substituent, they are increasingly being viewed as products of gene families that encode the different core proteins. Proteoglycans are found predominantly in the extracellular matrix (ECM) or associated with the cell surface of most eukaryotic cells where they bind to other matrix- and cell-associated components and growth factors (Bandtlow and Zimmermann, 2000; Funderburgh, 2000; Blackhall *et al.*, 2001; Filmus, 2001; Kresse and Schonherr, 2001). The interactive ability of proteoglycans derives from the chemical and structural diversity of either (or both) the polysaccharide or core protein components.

There has been significant progress in the molecular characterization of several proteoglycans as well as in the identification of novel family members, localizations, and biological functions. The importance of proteoglycans as constituents of the ECM and cell surface milieu is illustrated by the drastic change in their expression during development of several tissue systems and in certain disease processes, most notably heritable disorders of the skeleton known as chondrodysplasias. Although more than 150 different forms of chondrodysplasia have been described (Spranger, 1992), the genetic bases of relatively few have been identified as proteoglycan defects. Those elucidated are providing a better understanding of the role of proteoglycans in the development and maintenance of the cartilaginous skeletal elements.

Proteoglycan properties

Structure

The proteoglycans are composed of GAG chains consisting of repeated disaccharides, which usually contain a sulfated hexosamine and uronic acid, covalently linked to a central protein core (Figure 1). Type, size, and composition of GAG chains, primary sequence and domain arrangement of the protein core, or degree of substitution and distribution of the GAG chains along the protein core may all vary, leading to proteoglycan structures that are complex and diverse. Hybrid molecules with additional structural diversity may arise by substitution with N- and O-linked glycoprotein-type oligosaccharides or by having more than one type of GAG chain attached to the same core protein. Although there are features common to the GAGs, six distinct classes are recognized based on differences in monosaccharide composition, sulfation, and epimerization of the uronic acid. Four GAG classes—chondroitin sulfate, dermatan sulfate, heparin, and heparan sulfate—are linked to serines of the protein core via a common tetrasaccharide (xylose-galactose-galactose-glucuronic acid). Several excellent reviews of proteoglycan and GAG structures exist (Ruoslahti, 1988; Oldberg *et al.*, 1990; Kjellen and Lindahl, 1991; Wight *et al.*, 1991; Iozzo, 1998; Schwartz, 2000a,b; Esko and Lindahl, 2001).

A better understanding of the diversity of proteoglycan structure and function is emerging from the recent cloning of more than 40 full-length cDNAs encoding proteoglycan core proteins and the development of a system for classifying

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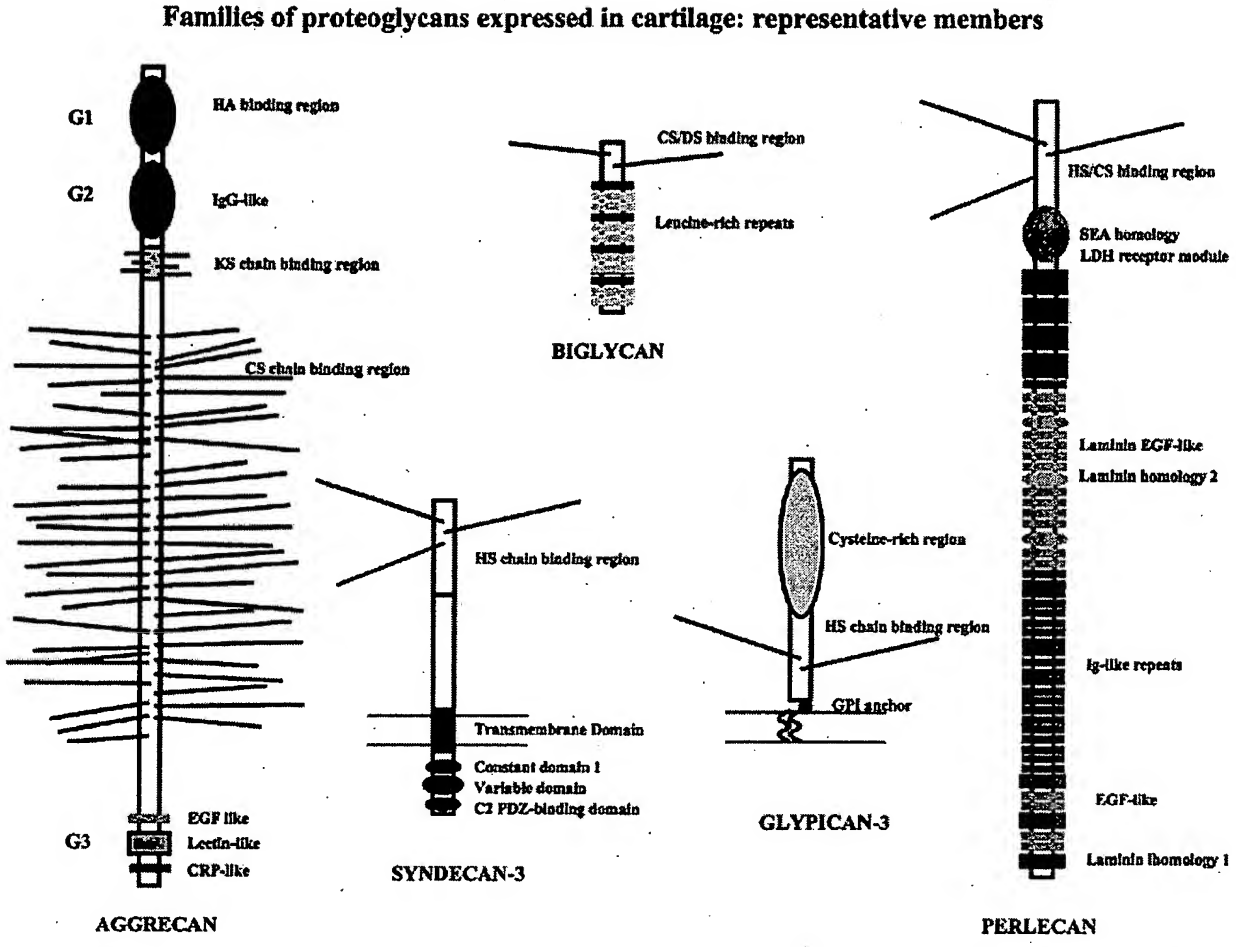

Fig. 1. Structures of representative members of each proteoglycan family expressed in cartilage. (For further domain structure details see the following reviews: Schwartz *et al.*, 1999; Bandtlow and Zimmermann, 2000; Matsushima *et al.*, 2000; Ahmad, 1998).

proteoglycans into gene families (Hassell *et al.*, 1986; Iozzo and Danielson, 1999; Schwartz *et al.*, 1999; Schwartz, 2000b). The concept of modular proteoglycans composed of discrete structural and functional domains, including both carbohydrate-attachment and carbohydrate-free regions (Figure 1), has evolved from analysis of deduced primary structures. For instance, the aggrecan gene family consists of four distinct proteoglycans—aggrecan, versican, neurocan, and brevican. The prototype for this family, aggrecan, has a structural organization consisting of two N-terminal globular domains (G1 and G2), one of which binds hyaluronan, and a C-terminal multifunctional binding domain (G3), part of which is lectin-like, separated by a variable-length carbohydrate-rich domain. Although varying in size and sequence all four members share this general organization, leading to their designation as hyalectins, that is, proteoglycans with hyaluronan- and lectin-interacting domains.

Several other families of proteoglycans are known (Figure 1), for instance, the cell-associated proteoglycans comprising the membrane-bound syndecan family, which have a short C-terminal cytoplasmic domain and a large extracellular domain substituted with heparan and chondroitin sulfate chains. The basement membrane proteoglycan, perlecan, also has a large, modular structure consisting of five major domains with multiple functions and a single N-terminal heparan sulfate attachment domain. The small leucine-rich proteoglycans are typified by the dermatan/chondroitin sulfate–substituted decorin and biglycan and the keratan sulfate–substituted fibromodulin and lumican. The variation within and among these distinct gene families, based on modular core protein organization and diversity in GAG type, provides a vast combinatorial potential for functional specificity that has been exploited by nature (Ruoslahti, 1988; Iozzo and Danielson, 1999; Schwartz, 2000b; Schwartz *et al.*, 1999).

Function

Most often, proteoglycans act as molecular organizers of the ECM and promoters of cell adhesion (Ruoslahti and Yamaguchi, 1991). Examples of this important role are numerous and include the large electron-dense aggregates characteristic of cartilage ECM (Hascall, 1977). The functional interactions that lead to these multimolecular aggregates

involve the unique terminal domains of the aggrecan core protein, which interact noncovalently with other matrix constituents (i.e., hyaluronan, type II collagen), thereby interconnecting the ECM and constituents of the cell surface. Members of the low-molecular-weight, leucine-rich proteoglycan family (i.e., decorin and fibromodulin) also participate in organizing the ECM by binding types I and II collagen (Ezura *et al.*, 2000; Svensson *et al.*, 2000).

Proteoglycans fulfill a variety of other biological functions, such as molecular concentration, growth modulation, ionic filtration, and biomechanical lubrication (Bandtlow and Zimmermann, 2000; De Cat and David, 2001; Knudson and Knudson, 2001; Rapraeger, 2001). Spatial immobilization of growth factors and cytokines may be one of the most important functions of proteoglycans. In this role, cell surface heparan sulfate proteoglycans (HSPGs) bind growth factors like fibroblast growth factor, which serves to protect the growth factors from degradation in the extracellular milieu, sequester a concentrated surface reservoir of growth factor (released only by degradation of the proteoglycan), or act as coreceptor to alter the conformation of the growth factor, thereby facilitating binding to its receptor and triggering of signal transduction pathways (Ruoslahti and Yamaguchi, 1991).

Biosynthesis

All GAGs (with the exception of hyaluronan) are synthesized as components of proteoglycans. Chain initiation for chondroitin sulfate, dermatan sulfate, heparan sulfate, and heparin begins with addition of xylose to a serine hydroxyl embedded in a specific peptide sequence (Bourdon *et al.*, 1987; Esko and Zhang, 1996), catalyzed by the chain-initiating xylosyltransferase (Schwartz, 1995). Elongation of the tetrasaccharide linkage region is catalyzed by distinct glycosyltransferases, each specific with respect to acceptor, donor and linkage formed. The chondroitin sulfate repeating polymer is then synthesized by the concerted action of an N-acetylgalactosaminyltransferase and a glucuronosyltransferase, concomitant with sulfation of the GAG chains either the 4 or 6 position of the hexosamine. (Rodén and Schwartz, 1975; Schwartz, 2000a; Sugahara and Kitagawa, 2000). The relative simplicity of chondroitin sulfate synthesis is in contrast to that of heparin or heparan sulfate, which require the concerted action of several additional modifying enzymes, many of which have now been cloned. These include the N-deacetylase/N-sulfotransferase, the glucuronic acid C-5 epimerase, the iduronic acid 2-0-sulfotransferase, and the glucosamine 6-0- and 3-0-sulfotransferases (Esko and Lindahl, 2001). Presumably, coordination between chain elongation and modification reactions leads to the regulated diversity of the heparan sulfates synthesized by different cells and tissues (Lindahl *et al.*, 1998).

The entire GAG complement is assembled while the proteoglycan core protein substrate is traversing the intracellular secretory pathway. Most insights into the dynamic and topological aspects of GAG synthesis have resulted from studies on the aggrecan system (Schwartz, 1995, 2000a; Luo *et al.*, 2000). In the endoplasmic reticulum (ER), N-linked oligosaccharides are added cotranslationally to the nascent core protein, whereas chondroitin sulfate chains are initiated by xylose addition after complete extrusion into the lumen of the ER. The xylosylated precursor core protein is translocated to early compartments of the Golgi for further modification reactions and then moved through the secretory pathway, yielding a fully glycosylated and sulfated aggrecan molecule (Kearns *et al.*, 1993; Vertel *et al.*, 1994). Biosynthetic studies for other members of the aggrecan gene family and other types of proteoglycans indicate that many aspects of GAG synthesis and assembly onto the various core proteins are similar to those elucidated for aggrecan (Sugahara and Kitagawa, 2000).

Proteoglycan expression during cartilage development

During skeletal development different proteoglycans are expressed in a highly defined pattern that is regulated spatially and temporally. Versican, a large chondroitin sulfate proteoglycan (CSPG), is expressed in the undifferentiated mesenchymal cells of the early limb bud and during the onset of prechondrogenic condensation, then disappears with the differentiation to cartilage (Kimata *et al.*, 1986; Shinomura *et al.*, 1993). This process is inversely correlated with a dramatic upregulation of the cartilage-specific CSPG, aggrecan, during the establishment and maturation of the chondrocytic phenotype (Schwartz *et al.*, 1993).

The process of cartilage formation, chondrogenesis, begins with the outgrowth of limb buds early in embryogenesis. Differentiation of limb bud commences with condensation of mesenchymal cells to form a cartilage primordium, which initiates the secretion of cartilage-specific ECM components. After a period of rapid cell proliferation, the chondrocytes in the center of these cartilaginous elements exit the cell cycle and differentiate to hypertrophic chondrocytes. These chondrocytes undergo apoptosis as their matrix is degraded by the invading vascular tissue, which introduces osteoblasts to initiate bone matrix formation. Replacement of cartilage by bone (ossification) proceeds in a highly organized fashion, requiring the orderly progression of several distinct cell phenotypes found within the growth plate (Mundlos and Olsen, 1997). The roles of several growth and transcription factors in the chondrogenesis process and bone growth are being increasingly elucidated (i.e., Sox9, Sox5, Sox6, Indian hedgehog, parathyroid hormone–related protein, FGFs) (Sandell and Adler, 1999; de Crombrugghe *et al.*, 2000, 2001; Lefebvre *et al.*, 2001; Vortkamp, 2001; Swarthout *et al.*, 2002)

Chondrocytes also organize their pericellular matrix, composed of type II collagen and aggrecan, in tight association with their cell surface. Aggrecan molecules interact with filaments of hyaluronan to form proteoglycan aggregates, an interaction that is further stabilized by link protein. Hyaluronan also interacts with a cell surface receptor, CD44, which has been suggested to facilitate the assembly and retention of the aggrecan-rich matrix at the surface of chondrocytes (Knudson, 1993).

Although aggrecan represents the bulk of the proteoglycans expressed during endochondral differentiation, other types are also expressed by chondrocytes. Two small dermatan/CSPGs, biglycan (Krusius and Ruoslahti, 1986) and decorin (Fisher *et al.*, 1989), are found in fetal articular cartilage, but their roles in the development and maintenance of cartilage are not known. Another member of this family, proteoglycan-Lb, is expressed in a more localized pattern associated with hypertrophic chondrocytes in the ossifying area of cartilage, suggesting the possible participation of this proteoglycan in

osteogenic processes (Shinomura and Kimata, 1992). Bone has a low content of fibromodulin, a small keratan sulfate proteoglycan from the family of leucine-rich core proteins. HSPGs are also present in the developing limb at early stages before differentiation of chondrocytes from mesenchyme. The large basement membrane HSPG, perlecan, and syndecan are widely distributed in early limb mesenchyme at many sites in addition to basement membranes (Solursh and Jansen, 1988; Solursh *et al.*, 1990); later they are reduced in the regions destined for chondrogenesis and become localized in myogenic regions. Perlecan is also localized to developing cartilage, with a low level of expression in precartilaginous condensations and accumulation in cartilage primordia preceded by that of collagen type II (French *et al.*, 1999). Human articular chondrocytes also express low levels of glypican mRNA (Grover and Roughley, 1995).

The specific roles of HSPGs and assembled binding partners in cartilage development and growth are still largely unknown. In general, most of the information on proteoglycan function during skeletal development has come from studies of animal mutant phenotypes or human disorders involving mutations of proteoglycans, their biosynthetic enzymes, or regulatory growth and transcription factors.

CSPG gene defects

Nanomelia: a lethal chondrodystrophy of chickens

There are multiple potential loci where mutations may affect proteoglycan-based phenotypes, that is, genes for the core proteins, GAG-modifying enzymes, or other factors that affect biosynthesis and secretion. Chick embryos homozygous for the autosomal recessive gene nanomelia (*nm*) exhibit an extreme form of micromelia (Landauer, 1965) with reduced trunk and head sizes and gross skeletal abnormalities including extremely shortened, broad and malformed limbs that are often twisted away from the body in a cephalad direction (Figure 2). Phenotypic distinctions can be observed as early as E8 and then become exaggerated with time of development, for example, trunk length (base of the skull to tail) of the mutants decreases from approximately 68% that of normal (*wt*) embryos at E14 to approximately 50% of normal at E20.

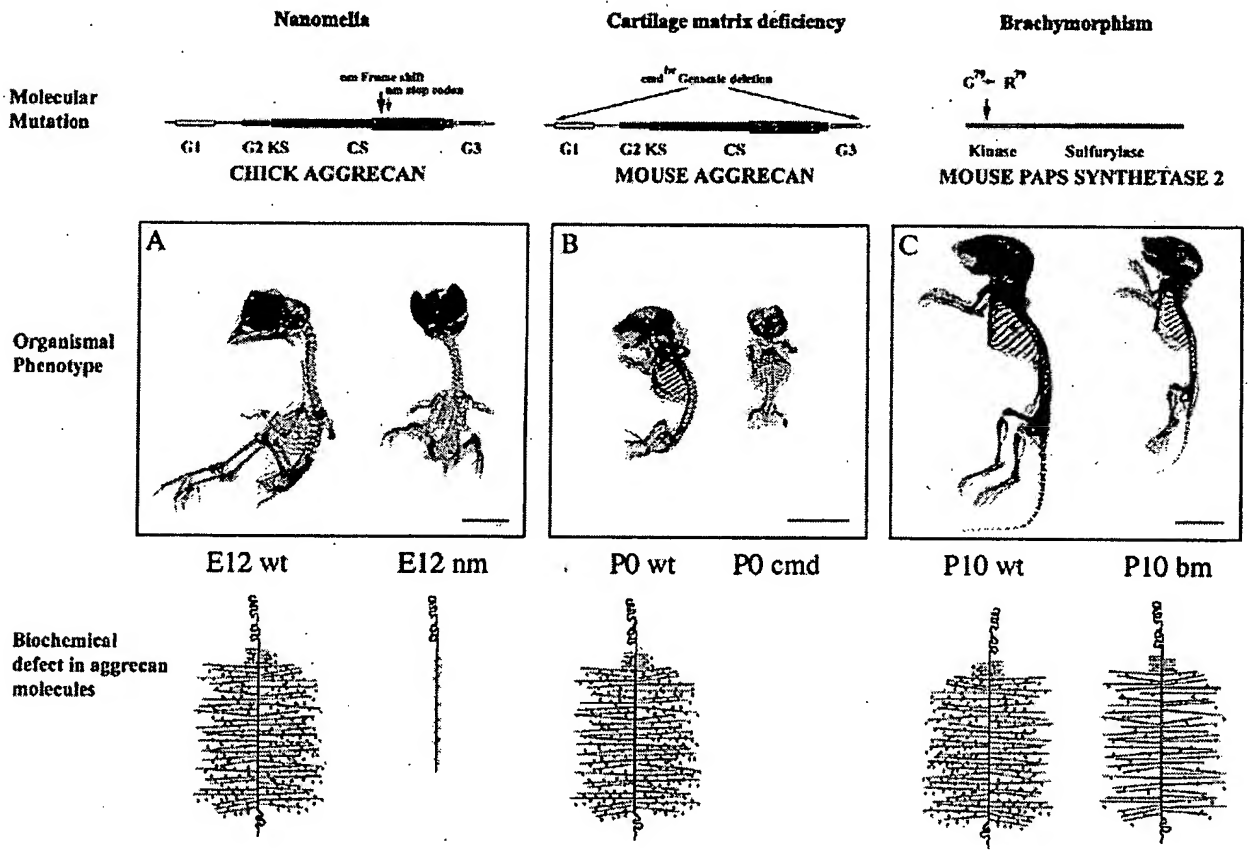

Fig. 2. Molecular mutations, biochemical defects, and organismal phenotypes in animal models with aggrecan-associated defects. Top panel presents diagram of protein domains with mutation sites indicated for aggrecan (A and B) and PAPS synthetase 2 (C). Middle panel shows calcified bone and cartilage differentially stained with Alzarin red and Alcian blue, respectively. (A) E14 wild-type and nanomelic chick mutant (*nm*). (B) Newborn (P0) wild-type and *cmd* mouse mutant. (C) P10 wild-type and *bm* mouse. Bar: 10 mm. Refer to Figure 1 for molecular description of aggrecan structure in bottom panel. S designates sulfate, and x designates xylose.

Reduction in extremity size during development is even more severe, suggesting a mechanism that involves significant growth retardation. In normal embryos the skeletal element growth plate zones are well organized, demarcated, and mature with extensive matrix elaboration and reduction in cell mass. In contrast, nanomelic embryos exhibit a rather homogeneous growth plate cell population, devoid of matrix and with complete loss of growth zone demarcation (Schwartz and Domowicz, 1998).

Early ultrastructural studies on the nanomelic mutant revealed a severe reduction in amount of matrix granules, normal-appearing collagen fibrils, and an overall decrease in extracellular space with resultant closer proximity of cells to each other (Pennypacker and Goetinck, 1976). The reduction in functional ECM is due to failure of nanomelic chondrocytes to produce the cartilage-specific CSPG, aggrecan, which interacts noncovalently with hyaluronan and link protein to form matrix (Pennypacker and Goetinck, 1976). Though nanomelic chondrocytes produce normal amounts of type II collagen and can synthesize chondroitin sulfate chains, aggrecan core protein is absent (Argraves *et al.*, 1981) and the amount of aggrecan core protein mRNA is significantly reduced (Stirpe *et al.*, 1987). The latter findings led to the hypothesis that the nanomelic mutation affects the regulation of transcription of the aggrecan gene (Stirpe *et al.*, 1987). However, in other studies a truncated form of aggrecan core protein was detected (O'Donnell *et al.*, 1988; Vertel *et al.*, 1993), which suggested that a mutation at or near the carboxy-terminal boundary of the CS-rich domain might account for the shortened core protein product.

Following the cloning and determination of the full-length aggrecan core protein cDNA sequence (Li *et al.*, 1993), the molecular basis for the truncated version of the core protein synthesized by nanomelic chondrocytes was identified (Table I) (Figure 2). Examination of the coding sequence and domain structure predicted that the mutation resulting in the shortened core protein is in the vicinity of the unique 20-amino-acid repeat region, C-terminal to the monoclonal antibody S103L binding site. Comparison of the normal and nanomelic cDNA sequences revealed that a single-base change at nucleotide 4553 (a G → T transversion) forms a premature stop codon in place of the normal glutamate codon and results in production of the truncated aggrecan precursor found in the mutant (Li *et al.*, 1993). The presence of this mutation in the nanomelic chick genome was confirmed by observation of a new restriction enzyme cutting site generated by the nucleotide substitution, a marker that can also be used to distinguish nanomelic and normal DNA in genotyping embryos prior to phenotypic recognition (Domowicz *et al.*, 1995).

It is interesting that the nanomelic mutation, which causes a shortened core protein precursor, essentially missing only the C-terminal G3 domain, does not result in the eventual secretion of a truncated version of aggrecan, rather than the observed complete absence of any form of aggrecan in the ECM. Early insights into this unexpected phenotype came from studies indicating that the truncated precursor remains in the ER and is not translocated to the Golgi for further processing like its normal counterpart (Vertel *et al.*, 1994). While residing in the ER, the nanomelic core protein is fully competent to become xylosylated and N-glycosylated, but unless mixed with Golgi glycosyltransferases via brefeldin treatment it remains unglycosylated. More recent work identified a portion of the C-terminal, G3 C-lectin globular domain encoded by exon 15 that is responsible for (1) translocation of aggrecan from the ER to the Golgi, (2) secretion from the cell, (3) galactosylation of CS chains, and (4) generation of Ca^{2+}-dependent galactose binding ability in the G3 domain (Domowicz *et al.*, 2000). Furthermore, in the absence of this subdomain there is excess accumulation of expression products in the ER (Luo *et al.*, 1996; Chen *et al.*, 2002), leading to a stress-related response involving certain chaperones, in turn followed by degradation via a ubiquitin-proteosome pathway (Domowicz *et al.*, 2000). In addition to explaining the essentiality of the G3 domain for aggrecan folding and maturation, this was the first report of the mode of degradation of misfolded proteoglycans and places the nanomelic mutation in the important category of processing/folding abnormalities, which are increasingly being found to be responsible for genetic diseases (Denning *et al.*, 1992).

Furthermore, the nanomelic mutation is expressed in nonskeletal tissue in the notochord (Domowicz *et al.*, 1995; Pettway *et al.*, 1996), and brain (Krueger *et al.*, 1992; Domowicz *et al.*, 1996; Li *et al.*, 1996; Schwartz *et al.*, 1996), and presumably in neural crest–derived cartilage (McKeon and Goetinck, 1979) and in membranous bone (Wong *et al.*, 1992). The broad tissue distribution of this ECM component may partly explain the severity of the aggrecan mutation in the developing organism. In this context therefore, the nanomelic mutant also provides a valuable knockout model for elucidating the function of aggrecan in these other tissues.

The nanomelic mutants are morphologically indistinguishable from wild type prior to embryonic day E8, and the fact that the numbers of skeletal elements has not changed indicates that this mutation does not alter the early determinants of the pattern formation process (Johnson *et al.*, 1994; Tickle, 1999), but rather may modify the growth processes of individual elements. The morphological observations suggest several possible consequences of the matrix deficit leading to the disorganized growth plate in the nanomelic mutant; for example, normal chondrocyte differentiation may be altered because aggrecan may be a key factor in controlling maturation, or the process of bone formation may be accelerated because aggrecan acts as an antiangiogenic factor in the matrix. More detailed analysis of cartilage differentiation and vascularization in the mutant is necessary to address these possibilities.

Cartilage matrix deficiency, a lethal chondrodystrophy of mice

Cartilage matrix deficiency (*cmd*) is an autosomal recessive lethal mutation in mice that causes short limbs and snout, enlarged abdomen, protruding tongue, and cleft palate in newborns (Rittenhouse *et al.*, 1978) (Figure 2). Homozygotes die at birth, presumably due to respiratory failure related to pulmonary hypoplasia. Mutant limb cartilage shows failure of chondrocyte column formation and lacks demarcation of the usually distinct resting, proliferative, and hypertrophic zones of epiphyseal cartilage. The chondrocytes are tightly packed with very little ECM between the cells in limb and trachea, similar to the nanomelic cartilage phenotype (Schwartz and Domowicz, 1998).

Matrix components, such as type II collagen, small proteoglycans, hyaluronan, and link protein are synthesized at normal rates, although the distribution of collagen is uneven with closely packed fibrils (Kimata *et al.*, 1981, 1984; Brennan

Table I. Skeletal defects related to proteoglycan structure in animal models and humans disorders

Disorder	Symbol	Phenotype	Biochemical defect	Gene affected	Reference[a]
Cartilage matrix deficiency (*cmd*/*cmd*-Bc)	Agc^{cmd} $Agc^{cmd\text{-}Bc}$	Short limb and snout, enlarged abdomen, protruding tongue, cleft palate, embryonic lethal	Aggrecan deficient matrix	Aggrecan	Watanabe *et al.*, 1994; Krueger *et al.*, 1999
Nanomelia (*nm*)	AGC1*nm	Micromelia, short, broad, malformed limbs and reduced trunk, embryonic lethal	Aggrecan deficient matrix	Aggrecan	Li *et al.*, 1993
Brachymorphism (*bm*)	$Papss2^{bm}$	Dome-shaped skull, shortened but not widened limbs, short tail	Reduced levels of PAPS; undersulfated proteoglycans	PAPS synthetase 2	Kurima *et al.*, 1998
Spondylo-epimetaphyseal dysplasia	SEMD	Short and bowed lower limbs, enlarged knee joints, early onset of degenerative joint disease	—	PAPS synthetase 2	ul Haque *et al.*, 1998
Diastrophic dysplasia	DTD	Short stature and generalized joint dysplasia	Defective sulfate transport; undersulfated proteoglycans	DTDST	Hästbacka *et al.*, 1994
Atelosteogenesis type II	AOGII	Micromelia, perinatally lethal	Defective sulfate transport; undersulfated proteoglycans	DTDST	Hästbacka *et al.*, 1996a
Achondrogenesis type 1B	ACG-1B	Short extremities and trunk, perinatally lethal	Defective sulfate transport; undersulfated proteoglycans	DTDST	Superti-Furga *et al.*, 1996
Simpson-Golabi-Behmel syndrome	SGBS	Multiple tissues overgrowth; skeletal abnormalities; embryonal tumors	Reduced levels of heparan sulfate at cell surface	Glypican-3	Pilia *et al.*, 1996; Veugelers *et al.*, 2000
Hereditary multiple exostoses	HMX	Juxtaepiphysial exostoses, skeletal malformation, short stature	Defective heparan sulfate biosynthesis	EXT-1, EXT-2	Raskind *et al.*, 1998; Wolf *et al.*, 1998; Wuyts *et al.*, 1998; Park *et al.*, 1999
Dyssegmental dysplasia, Silverman-Handmaker type	DDSH	Micromelia, anisospondyly, flat face, short and bent long bones	Reduced perlecan staining in matrix	HSPG-2/ Perlecan	Arikawa-Hirasawa *et al.*, 2001
Perlecan null-mouse	—	Exencephaly, cleft palate, perinatally lethal, short limb and abnormally bent vertebral column	Perlecan deficient matrix	Perlecan	Costell *et al.*, 1999

[a]The references correspond to the identification of the molecular bases for the listed disease.

et al., 1983). In contrast, the amount of aggrecan synthesized by *cmd* chondrocytes is greatly reduced (Kimata *et al.*, 1981, 1984; Brennan *et al.*, 1983). The *cmd* locus was localized to chromosome 7 (Kochhar, 1985), establishing a correlation with the aggrecan gene locus. A mutation within the coding region of the aggrecan gene in *cmd* mice was reported: a 7-bp deletion in exon 5 causes a frameshift and premature stop codon in exon 6, thereby producing a truncated core protein of approximately 32kD (Watanabe *et al.*, 1994) (Table I). *Cmd* heterozygotes show a mild dwarfism and develop spinal misalignment and degeneration of the vertebral disk with age (Watanabe *et al.*, 1997). These observations raise the possibility that predisposition to spinal degeneration in humans could be associated with lower dosage of aggrecan gene expression in cartilage.

Another spontaneous recessive mutation at the aggrecan locus on chromosome 7, *cmd-Bc*, occurring in the BALB/cGaBc background (Bell *et al.*, 1986), causes short limbs and snout, enlarged abdomen, protruding tongue, and cleft palate in newborns, a phenotype nearly identical to the *cmd*. Homozygotes can be recognized as early as E15 by reduced limb length and abnormal limb shape. *Cmd-Bc* is a large deletion, producing the complete loss of exons 2 to 18 in the aggrecan gene and resulting in a significantly shortened mRNA (1.2 kb) (Krueger

et al., 1999) (Table I). The mutation most likely was the consequence of a nonhomologous recombination event, as topoisomerase cleavage sites and 7-bp direct repeats flank the deletion splice site (Krueger *et al.*, 1999). Although these two allelic deletion mutations in the murine aggrecan gene have been identified, there are no reports thus far of any mutations in the human aggrecan gene (Finkelstein *et al.*, 1991) (which likely would be lethal), although numerous skeletal disorders have been reported in humans due to defects in other ECM molecules (Warman *et al.*, 1993; Prockop *et al.*, 1994). The lack of known counterparts in humans may reflect differing frequencies of "hot spots" for illegitimate recombination in the human and murine genes. Interestingly, a variable number of tandem repeat polymorphisms found in the CS-attachment region of the human aggrecan gene is associated with bilateral hand osteoarthritis (Doege *et al.*, 1997; Horton *et al.*, 1998).

In summary, lethal chondrodystrophies in both birds and mammals result from mutations in the gene that codes for the core protein of the cartilage-specific proteoglycan aggrecan. The phenotypes are nearly identical, with the total absence of aggrecan leading to severely reduced ECM, disruption of the columnar organization of the growth plate, markedly shortened and somewhat broader bones, and death at birth. Despite the differences in size and growth characteristics of certain limb skeletal elements (e.g., wings and biped legs in birds versus quadruped legs in mice), the role this gene product plays in development appears to be remarkably similar in both species.

Brachymorphism: a nonlethal growth disorder

In addition to chondrodystrophies associated with mutations in the aggrecan core protein gene, growth disorders caused by defects in biosynthetic pathways necessary for modification of aggrecan are increasingly being identified. One such nonlethal growth disorder is murine brachymorphism (*bm*), characterized by dome-shaped skull, short thick tail, and shortened but not widened limbs (Figure 2) (Lane and Dickie, 1968; Schwartz *et al.*, 1978; Sugahara and Schwartz, 1979, 1982a,b; Schwartz and Domowicz, 1998). The phenotype is inherited as an autosomal recessive and *bm* homozygotes breed normally, have life spans comparable to wild-type mice, and are about the same size as normals at birth. However, a difference in *bm* mutant size becomes apparent over the first 4 weeks of life, eventually resulting in a 50% reduction in limb length and a 25% reduction in axial skeletal size compared to normal mice. The effect on growth is concomitant with a progressive reduction in size of the columnar and hypertrophic zones in the epiphyseal growth plates without disruption of the zonal organization. Histological and ultrastructual studies suggest a defective cartilage matrix that contains normal collagen fibrils but proteoglycan aggregate granules that are smaller than normal and present in reduced numbers, particularly in the growth plate (Orkin *et al.*, 1977). Biochemical analysis showed that *bm* cartilage contains normal levels of GAGs that are significantly undersulfated. The reduced incorporation of sulfate in bm cartilage stems from limited activation of sulfate (3′-phosphoadenyl-5′-phosphosulfate, PAPS), predominantly due to a reduction in APS-kinase activity (Schwartz *et al.*, 1978; Sugahara and Schwartz, 1979, 1982a,b).

Genetic linkage studies localized the *bm* gene on mouse chromosome 19 (Lane and Dickie, 1968; Rusiniak *et al.*, 1996), but the gene responsible for the brachymorphic defect was not identified until the existence of a PAPS synthetase gene family was recognized (Li *et al.*, 1995; Kurima *et al.*, 1998; Schwartz, 2002). Two murine members of the PAPS synthetase family, SK1 and SK2, have been identified. SK2 is on chromosome 19, tightly linked with the marker for the *bm* locus. Sequence analysis of *bm* SK2 cDNA revealed a missense mutation that results in a glycine-to-arginine substitution at a highly conserved portion of the APS kinase domain (Li *et al.*, 1995; Kurima *et al.*, 1998, 1999) (Table I, Figure 2). Expressed *bm* SK2 failed to catalyze the APS kinase reaction or to synthesize PAPS, confirming that the primary defect responsible for the *bm* phenotype resides in PAPS synthetase isoform 2.

Because PAPS is the universal sulfate donor for synthesis of all naturally occurring sulfated compounds, it was initially surprising that the PAPS synthesis defect in the *bm* mouse produced only a skeletal disorder and not a more severe, generalized phenotype. There are other tissues where a significant demand for PAPS might be predicted, for instance, liver, which is rich in heparan sulfate and uses sulfoconjugation for detoxification; skin, which is rich in dermatan sulfate; or kidney and brain, which have high concentrations of sulfatides. The tissue specificity of the *bm* defect (Sugahara and Schwartz, 1982b) correlates with the tissue-specific localization of the PAPS synthetase isozymes, with the SK2 form bearing the *bm* mutation being more highly expressed in cartilage and less prevalent in brain and skin.

Unlike the aggrecan core protein mutations that have no counterpart in humans, the identification of the PAPS synthetase 2 isoform mutation in the *bm* mouse (Kurima *et al.*, 1998, 1999) was followed by the elucidation of an SK2 mutation in human spondyloepimetaphyseal dysplasia (ul Haque *et al.*, 1998) (Table I). This disorder is characterized by short and bowed lower limbs, enlarged knee joints, and early onset of degenerative joint disease in the hands and knees (Ahmad *et al.*, 1998). Clearly the human and murine PAPS synthetase defects underscore the importance of proper sulfate metabolism for cartilage development and skeletal growth.

Other sulfation defects

Several other human genetic disorders associated with defects in transport of sulfate into the cell also lead to undersulfated proteoglycans and chondrodysplasias (Hästbacka *et al.*, 1996b) (Table I). Three distinct recessive chondrodysplasias of different severity are caused by mutations in the same sulfate transporter gene, *DTDST*, which codes for a novel plasma membrane sulfate transporter. Patients with the first of these to be characterized, diastrophic dysplasia (DTD), exhibit disproportionately short stature and generalized joint dysplasia but usually have a normal life span (Hästbacka *et al.*, 1994). Subsequently, atelosteogenesis type II, a rare recessive perinatally lethal chondrodysplasia (micromelia) that is phenotypically similar to DTD, was also shown to be caused by mutations in the *DTDST* gene (Hästbacka *et al.*, 1996a). Achondrogenesis type 1B (ACG-1B), a lethal chondrodysplasia characterized by extremely short extremities and short trunk, was first mistakenly categorized as a defect in the sulfate-activation enzymes (Superti-Furga, 1994), but later found to be allelic to DTD (Superti-Furga *et al.*, 1996). The human and animal models with sulfation mutations clearly highlight the importance of this posttranslational modification to the functioning of

proteoglycans in skeletal tissue and the multiplicity of genes that might be affected in sulfate uptake, activation, and utilization.

With respect to proteoglycan disorders in general, it is curious that only human chondrodystrophies involving this very late posttranslational modification (sulfation) have been identified. The range of severity observed for disorders (from the lethal ACG-1B to the intermediate atelosteogenesis type II to the less severe DTD) resulting from mutations in the same sulfate transporter gene suggests that the amount of residual transport activity produced by the affected proteins may be responsible for the modulated expression observed clinically. Nonetheless, limitation in sulfate uptake into the cell, which presumably leads to undersulfated aggrecan, can under some circumstances, for example, ACG-1B, be lethal. Therefore it may not be surprising that mutations affecting production of the human aggrecan core protein (analogous to the nanomelic chick or *cmd* mouse) have not emerged and/or survived long enough to be detected (Finkelstein *et al.*, 1991).

Other CSPG-related defects

As more transcription modulators and growth factors that control aggrecan expression are identified, it will become possible to determine whether mutations in the genes for these regulatory factors result in skeletal abnormalities. One candidate disorder is campomelic dysplasia, a congenital skeletal abnormality in humans with characteristics similar to those observed in the nanomelic and *cmd* animal mutants. Individuals with campomelic dysplasia exhibit shortening and bowing of the long bones and abnormal facial features, including macrocephaly, micrognathia, cleft palate, and flat nasal bridge, as well as sex reversal (Mansour *et al.*, 1995). Affected neonates usually die due to respiratory insufficiency. In most of the cases analyzed this disorder appears to be caused by mutations in the *SRY*-related gene *SOX9* (Wagner *et al.*, 1994), a member of a large family of developmentally regulated genes coding for transcription factors. *SOX9* in particular is expressed in the mesenchymal condensations prior to cartilage formation, and its expression is maintained in perichondrium and chondrocytes of the resting, proliferative, and upper hypertrophic zones (Wagner *et al.*, 1994). Thus this transcriptional activator may play a role in establishing and maintaining the chondrocytic phenotype, perhaps by controlling cartilage-specific genes like types II and XI collagen and aggrecan (Bridgewater *et al.*, 1998; Bi *et al.*, 1999; de Crombrugghe *et al.*, 2000; Huang *et al.*, 2001).

Mouse mutants with other chondroitin/dermatan sulfate proteoglycan defects also have growth abnormalities; biglycan-null mice are born without patterning or growth defects, but the effect on skeletal growth rate appears 3 months after birth, characterized by a decrease in bone mass and in trabecular bone formation (Xu *et al.*, 1998). This osteoporosis-like phenotype implicates biglycan in the process of bone formation and highlights the importance of lower-abundance CSPGs in skeletal development as well.

HSPG gene defects

Mutations have also been described in HSPGs that are associated with skeletal growth disorders although not classified as chondrodystrophies. Of the two families of cell surface HSPG—that is, the syndecan-like proteoglycans that span the cell membrane or the glypican-like proteoglycans that are linked to the cell surface via glycosylphosphatidyl inositol (David, 1993) (Figure 1)—mutations in *GPC3*, a glypican gene, have been shown to be responsible for the multifaceted, X-linked Simpson-Golabi-Behmel syndrome (SGBS) (Pilia *et al.*, 1996; Veugelers *et al.*, 2000), which is characterized by pre- and postnatal overgrowth, coarse face, visceral and skeletal anomalies, and increased risk of embryonal tumors (Neri *et al.*, 1998) (Table I). Thus far, the mechanism underlying the overgrowth phenotype in this syndrome is poorly understood. Interestingly, the *Drosophila* mutant gene *dally* encodes a protein belonging to the glypican family of cell-surface HSPGs (Nakato *et al.*, 1995) and is required in cell cycle control for proper morphogenesis of several tissues; in both fly and human phenotypes a derangement of cellular growth control is suggested. Furthermore, among all the mutations of *GPC3* found in SGBS patients at least one, a W296R missense mutation, affects heparan sulfate substitution on the core protein, highlighting the key role of heparan sulfate in glypican function. (Paine-Saunders *et al.*, 2000; Veugelers *et al.*, 2000)

Some intriguing mutations that alter GAG biosynthesis have also been identified in *Drosophila*. *Sugarless* (*sgl*) encodes a protein homologous to vertebrate UDP-glucose dehydrogenase, which generates the UDP-glucuronic acid used for synthesis of chondroitin/dermatan sulfate, heparan sulfate, heparin, and hyaluronan. Mutations in *sgl* suggest a role for *sgl* in the wingless (*Wg*) and decapentaplegic (*Dpp*) signaling pathways (Binari *et al.*, 1997; Hacker *et al.*, 1997; Haerry *et al.*, 1997). Another gene that disrupts *Wg* signaling, *sulfateless* (*sfl*), encodes a protein homologous to the heparan sulfate–modifying enzyme GlcNAc N–deacetylase/sulfotransferase (Lin and Perrimon, 1999). Last, mutants with defects in the *Drosophila* gene *tout-velu* (*ttv*) exhibit a reduction in heparan sulfate but not chondroitin sulfate synthesis, and these mutations affect embryonic patterning by interfering with hedgehog signaling. *Ttv* encodes a homolog of mammalian EXT1 which, like EXT2, is a glycosyltransferase involved in the biosynthesis of heparan sulfate chains (Lind *et al.*, 1998; Lin *et al.*, 2000; Senay *et al.*, 2000). EXT-1 can restore activity of copolymerase, the enzyme that catalyzes transfer of glucuronic acid and N-acetylglucosamine to growing heparan sulfate chains in deficient cell lines (Lind *et al.*, 1998; Wei *et al.*, 2000). Mutations in human EXT1 and EXT2 are responsible for hereditary multiple exostoses, an autosomal dominant skeletal disorder characterized by the formation of cartilage-capped exostoses and short stature (Raskind *et al.*, 1998; Wolf *et al.*, 1998; Wuyts *et al.*, 1998; Park *et al.*, 1999) (Table I). EXT1-knockout mice die at embryonic day 8.5 due to defects in mesoderm and extra-embryonic tissue formation. Heterozygous mice show a 10% loss of bone length but not the exostoses phenotype (Lin *et al.*, 2000). Taken together, these results suggest that an HSPG (or more than one) may be involved in hedgehog function in growth plate development, leading to skeletal disorders; however, the identity of this HSPG(s) remains unknown.

Studies in mice with homozygous null mutations in the perlecan gene (Costell *et al.*, 1999) led to the elucidation of the molecular basis of a rare human autosomal recessive skeletal dysplasia, dyssegmental dysplasia, Silverman-Handmaker type (DDSH) (Arikawa-Hirasawa *et al.*, 2001) (Table I). Perlecan-null mouse embryos exhibit deteriorating basement

membrane in regions of increased mechanical stress during development, as well as skeletal defects characterized by disproportionate dwarfism, disorganized growth plate, cleft palate, and perinatal lethality. Shortened collagen fibrils, reduced fibrillar network, and elevated expression of cartilage ECM genes are characteristic of perlecan-null embryo chondrodysplasia (Costell *et al.*, 1999). Studies on the perlecan gene from individuals with DDSH, who share many of the phenotypic characteristics of the perlecan-null mice, have identified a frame-shift mutation resulting in a truncated protein core that is not secreted (Arikawa-Hirasawa *et al.*, 2001) (Table I).

Summary

In summary, the recent identification of the molecular basis of hereditary skeletal disorders associated with proteoglycan gene mutations in humans and in some of the long-standing animal models is providing critical insight into the roles of proteoglycans in skeletal development and growth. Identification of additional components in the pathways directly involved in synthesis of these complex macromolecules should provide more candidate genes for study. Also important will be the elucidation of upstream signaling pathways that are responsible for transcriptional regulation of the proteoglycan genes directly, as well as the growth factors, cytokines, and receptors that influence both the early processes of mesenchymal condensation and chondrocyte differentiation, as well as later steps, such as growth plate maturation.

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Abbreviations

ACG-1B, achondrogenesis type 1B; CSPG, chondroitin sulfate proteoglycan; DDSH, dyssegmental dysplasia, Silverman-Handmaker type; DTD, diastrophic dysplasia; ECM, extracellular matrix; ER, endoplasmic reticulum; GAG, glycosaminoglycan; HSPG, heparan sulfate proteoglycan; PAPS, phosphoadenosine phosphosulfate; SGBS, Simpson-Golabi-Behmel syndrome.

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